A Hydrophobic Domain in the Large Envelope Protein Is Essential for Fusion of Duck Hepatitis B Virus at the Late Endosome

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The duck hepatitis B virus (DHBV) envelope is comprised of two transmembrane (TM) proteins, the large (L) and the small (S), that assemble into virions and subviral particles. Secondary-structure predictions indicate that L and S have three α-helical, membrane-spanning domains, with TM1 predicted to act as the fusion peptide following endocytosis of DHBV into the hepatocyte. We used bafilomycin A1 during infection of primary duck hepatocytes to show that DHBV must be trafficked from the early to the late endosome for fusion to occur. Alanine substitution mutations in TM1 of L and S, which lowered TM1 hydrophobicity, were used to examine the role of TM1 in infectivity. The high hydrophobicity of the TM1 domain of L, but not of S, was shown to be essential for virus infection at a step downstream of receptor binding and virus internalization. Using wild-type and mutant synthetic peptides, we demonstrate that the hydrophobicity of this domain is required for the aggregation and the lipid mixing of phospholipid vesicles, supporting the role of TM1 as the fusion peptide. While lipid mixing occurred at pH 7, the kinetics of insertion of the fusion peptide was increased at pH 5, consistent with the location of DHBV in the late-endosome compartment and previous studies of the nonessential role of low pH for infectivity. Exchange of the TM1 of DHBV with that of hepatitis B virus yielded functional, infectious DHBV particles, suggesting that TM1 of all of the hepadnaviruses act similarly in the fusion mechanism.

In order to initiate productive cell infection, enveloped viruses need to fuse to a host cell membrane in order to release a capsid into the cytoplasm. Details of this process have been well described for viruses such as the influenza A virus, human immunodeficiency virus, or tick-borne encephalitis virus. However, details of the virus fusion process in the Hepadnaviridae, a family of enveloped viruses that replicate in the liver, remain unknown. Studies of the early infection steps of hepatitis B virus (HBV) have been hampered by the lack of a suitable infection system. However, the study of duck hepatitis B virus (DHBV), which provides a primary cell culture infection system, has enabled elucidation of some viral-entry mechanisms, most notably the identification of the attachment receptor, carboxypeptidase D (CPD) (15, 16, 33, 35).

The DHBV envelope is comprised of two transmembrane proteins, the large (L) and small (S) surface proteins, that assemble into virions and subviral particles (SVPs). These proteins are translated by differential initiation from a single pre-S/H9251-helical, membrane-spanning domain sequence which comprises the S protein. Secondary-structure predictions suggest that the S protein and C-terminal S domain sequence which comprises the S protein. The high hydrophobicity of the TM1 domain of L, but not of S, was shown to be essential for virus infection at a step downstream of receptor binding and virus internalization. Using wild-type and mutant synthetic peptides, we demonstrate that the hydrophobicity of this domain is required for the aggregation and the lipid mixing of phospholipid vesicles, supporting the role of TM1 as the fusion peptide. While lipid mixing occurred at pH 7, the kinetics of insertion of the fusion peptide was increased at pH 5, consistent with the location of DHBV in the late-endosome compartment and previous studies of the nonessential role of low pH for infectivity. Exchange of the TM1 of DHBV with that of hepatitis B virus yielded functional, infectious DHBV particles, suggesting that TM1 of all of the hepadnaviruses act similarly in the fusion mechanism.

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to viral fusion. In line with this phenomenon, the conformational change induced by low pH increased particle hydrophobicity, facilitating binding to membranes, through exposure of a previously hidden hydrophobic TM1 domain (Fig. 1E) (12). The possibility that a protease is also involved during entry was suggested by an earlier study showing the infection of the normally nonpermissive HepG2 cell line with HBV pretreated with V8 protease and incubated with cells at pH 5.5 (19, 20).

Although DHBV is endocytosed and may enter the acidic endosomal pathway, several earlier studies using agents which raise endosomal pH indicated that low pH does not appear to be essential for the initiation of hepadnaviral infection (14, 24). These studies concluded that DHBV may not be transported through an acidic compartment or may not require low pH for fusion. However, the agents used in these studies were unable to raise the pH of the late endosome to neutrality and our recent studies indicate that even brief treatment at a mild pH of 6.5 under reducing conditions causes a major conformational change in L (12). Thus, while not strictly essential, low pH may play a role in the kinetics of DHBV entry.

The candidate hepadnaviral fusion peptide was identified by the similarity of the N-terminal sequence of TM1 of HBV with the consensus sequence for fusion peptides (19). While confirmation of the potential of such sequences to act in fusion was shown by lipid mixing of synthetic peptides corresponding to the N terminus of DHBV and HBV TM1 with phospholipid vesicles (25–27), these synthetic peptide studies alone do not identify the domain as essential for DHBV infectivity.

In this study, we used the primary duck hepatocyte (PDH) infection system to examine whether DHBV enters the early or late endosome for fusion and whether the TM1 domain plays a part in this process. Using L and S mutants with reduced TM1 hydrophobicity, we show that the high hydrophobicity of the TM1 domain of L alone is essential for virus infection at a step downstream of receptor binding and virus internalization. Similar mutations in synthetic TM1 peptides were used to demonstrate that the hydrophobicity of this domain is required for the aggregation and lipid mixing of phospholipid vesicles, suggesting that this domain represents the fusion peptide of the virus. Moreover, the kinetics of aggregation and lipid mixing of phospholipid vesicles with the TM1 peptide is increased with low pH, pointing to a role for pH in the kinetics of DHBV entry.

**MATERIALS AND METHODS**

**Plasmids.** Plasmids used in this study are described in Table 1. The LT1.4 mutant was generated by substitution of a KpnI-BstEII fragment (DHBV DNA from nucleotides 1296 to 1847 [21]) in CDL-wt with the equivalent sequence from the mutant ST1.4, containing alanine substitutions in the TM1 (9). DHBV/HBV chimeras were created by the substitution of the DHBV TM1 sequence from nucleotides 1296 to 1847 with the equivalent sequence of the TM1 domain of L alone is essential for virus infection (14, 24). These studies concluded that DHBV may not be transported through an acidic compartment or may not require low pH for fusion. However, the agents used in these studies were unable to raise the pH of the late endosome to neutrality and our recent studies indicate that even brief treatment at a mild pH of 6.5 under reducing conditions causes a major conformational change in L (12). Thus, while not strictly essential, low pH may play a role in the kinetics of DHBV entry.

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**TABLE 1. Plasmids and their products used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDL-wt</td>
<td>Wild-type L protein (gift from H. Schaller, ZMBH, Germany)</td>
</tr>
<tr>
<td>pMT7DL-wt</td>
<td>Wild-type L protein (gift from H. Schaller, ZMBH, Germany)</td>
</tr>
<tr>
<td>pCI-S</td>
<td>Wild-type S protein</td>
</tr>
<tr>
<td>LTM1</td>
<td>L protein with deleted TM1 (aa 169–186)</td>
</tr>
<tr>
<td>ST1.4</td>
<td>S protein with alanine substitutions at positions 8, 15, 22, and 26</td>
</tr>
<tr>
<td>LT1.4</td>
<td>L protein with alanine substitutions at positions 169, 176, 183, and 187</td>
</tr>
<tr>
<td>1165A</td>
<td>Replication-competent RNA pregenome (without L protein)</td>
</tr>
<tr>
<td>1285C</td>
<td>Replication-competent RNA pregenome (without S protein)</td>
</tr>
<tr>
<td>L: DHTM1</td>
<td>Chimeric L protein carrying HBV TM1 sequence</td>
</tr>
<tr>
<td>L: DHTM1 Q177L</td>
<td>Chimeric L protein carrying HBV TM1 sequence with a Q177A mutation</td>
</tr>
</tbody>
</table>

*Numbers in parentheses are reference numbers.*
residue was mutated into Thr188, by using overlap extension PCR, to obtain a full HBV TM1 sequence.

Peptides. Three 16-mer peptides covering the N-terminal region of TM1 (aa 162 to 177 of L) were synthesized according to specifications (C-terminal amidation) by Genescript, Inc. The following peptides were synthesized: wild-type (WT) (MSGTFGGALAGLGLG), LT1.2 (MSGTFGGALAGLGLG), and scrambled WT (GMALLSGPGLITGGGL). Particles were determined by high-performance liquid chromatography by Genescript, Inc., to be 87.3%, 93.0%, and 75.8%, respectively, and cell transfections. Chicken hepatocyte LMH (Leghorn male hepatoma) cells, used for transfections, were maintained in Dulbecco's modified Eagle's medium-F-12 medium (Gibco) supplemented with 5% fetal bovine serum and 100 U/ml penicillin-streptomycin (Gibco). Cells were transfected using the DEAE-dextran method (11), with 35 μg of each plasmid DNA per 80-cm² culture flask (Nunc). PDH cultures were obtained by collagenase perfusion of 7-day-old Pekin-Aylesbury ducklings to be negative for DHBV, as described previously (24). Cells were seeded on 12-well plates (Nunc) with 12-mm coverslips at 1 × 10⁶ cells/ml and maintained in Williams E medium supplemented with 10 mM Tris (pH 7.6), 6 mM HEPES, 0.02% bicarbonate, 1% penicillin-streptomycin (Gibco), 0.02% glucose, 10 μM hydrocortisone 21-hemisuccinate (Sigma), 1 μg/ml insulin, and 1.5% dimethyl sulfoxide (DMSO).

SVP isolation and purification. LMH cells were cotransfected with plasmids encoding mutant or wild-type L and S proteins (LT1.4, L(S)), medium–F-12 medium (Gibco) supplemented with 5% fetal bovine serum and 100 U/ml penicillin-streptomycin (Gibco). Cells were transfected using the DEAE-dextran method (11), with 35 μg of each plasmid DNA per 80-cm² culture flask (Nunc). PDH cultures were obtained by collagenase perfusion of 7-day-old Pekin-Aylesbury ducklings to be negative for DHBV, as described previously (24). Cells were seeded on 12-well plates (Nunc) with 12-mm coverslips at 1 × 10⁶ cells/ml and maintained in Williams E medium supplemented with 10 mM Tris (pH 7.6), 6 mM HEPES, 0.02% bicarbonate, 1% penicillin-streptomycin (Gibco), 0.02% glucose, 10 μM hydrocortisone 21-hemisuccinate (Sigma), 1 μg/ml insulin, and 1.5% dimethyl sulfoxide (DMSO).

SVP iso...
fluorescence increase was measured at 2-s intervals for 5 min at 37°C by using a Fluorostar fluorometer (BMG Lab Technologies) with an excitation spectrum and emission spectra at 560 nm and 590 nm, respectively. After 5 min, Triton X-100 (Sigma) was added to a final concentration of 1% and the resulting fluorescence value was taken as 100% dequenching ($F_{100}$). The background fluorescence value before peptide addition was taken as 0% dequenching ($F_0$).

The extent of R18 dequenching at time $t$ was calculated according to the following equation:

$$\% \text{ R18 dequenching} = \frac{100(F_t - F_0)}{(F_{100} - F_0)}.$$

**RESULTS**

**DHBV enters the late-endosome compartment.** To confirm previous reports that the virus is endocytosed, we traced its uptake into PDHs along with a fluorescent transferrin conjugate by confocal microscopy. Transferrin is a cycling iron chelator protein that enters the cell via clathrin-dependent receptor-mediated endocytosis and is targeted to the endosomal pathway. Acidic conditions of the endosome result in iron release and return of transferrin to the cell surface via recycling endosome. Serum-derived DHBV particles and transferrin were bound to the cells by incubation at 4°C for 1 h. Internalization of transferrin and bound particles was initiated by shifting the temperature to 37°C and incubation for 2 h. Following incubation, the unbound material was washed off and cells were fixed and stained for DHBV L protein. Figure 2A shows that transferrin forms punctate clusters within the PDH cytoplasm (Fig. 2A). Although very little bound virus was observed, image overlays with DHBV show that serum-derived DHBV particles colocalize with transferrin presumably in the early and late endosome.

Bafilomycin A1 is a potent inhibitor of vacuolar proton ATPases and blocks trafficking from the early to late endosome. This proton pump is responsible for the acidification and establishment of the pH gradient in the endosome, which decreases from around pH 6.2 in the early endosome to pH 5.5 in the late endosome. To determine if DHBV enters the late endosome, bafilomycin A1 was added to primary duck hepatocytes at various time points during and after the incubation of the virus with the cells. Successful completion of infection was measured by immunofluorescence 5 days postinfection, a
time point before multiple rounds of infection from progeny virus have occurred. Addition of bafilomycin A1 from the beginning of the infection period completely blocked DHBV infectivity, whereas the addition at time points after infection showed an increase in the number of positive cells (an approximate doubling) from 2 h to 4 h to 8 h, with the number of positive cells at the 8-h time point similar to that with untreated cells. This indicates that DHBV transits through the late endosome and would be exposed to envelope-deforming conditions of that compartment (Fig. 2B).

Alanine substitutions of four hydrophobic residues in TM1 or deletion of TM1 does not affect assembly of virions or SVPs. Fusion peptides are 15- to 20-aa sequences that insert into cellular lipid membranes, allowing fusion of the viral lipid membrane in order to release the capsid into the cytoplasm of the cell. High hydrophobicity of the fusion peptide plays a crucial role in this process, and analysis of the hepadnaviral TM1 sequences in an α-helical wheel diagram reveals that one face of the helix consists of the hydrophobic residues leucine, isoleucine, or in the case of the mammalian hepadnaviruses also includes the residues phenylalanine or tryptophan (9). To determine if TM1 is involved in the DHBV fusion process, we used mutants where either TM1 was deleted (ΔTM1) or the hydrophobicity of TM1 was lowered by substitution of the four residues on the hydrophobic face (He165-Leu176-Leu183, and Leu187) with alanine (LT1.4) (Fig. 3B). While our previous studies indicate that only L undergoes a conformational change at low pH, the role of S either alone or in concert with L cannot be ruled out. Therefore, TM1 mutants were studied with both L and S independently by the use of constructs expressing either mutant L or S protein. However, the corresponding deletion mutant in S could not be used, as previous studies have shown that the presence of this domain is essential for the expression of the S protein (9).

To determine whether the mutants are able to express and assemble into SVPs, LMH cells were transfected with L- and S-encoding plasmids, so that mutant L could be coexpressed with wild-type S and vice versa. The cell membrane fractions from transfected cells were analyzed by Western blotting, and the assembly of SVPs was analyzed by sedimentation of particles from the cytosolic fraction through 20% sucrose, as described previously (10). All mutants were expressed and assembled into SVPs (Fig. 3C). Previous studies have shown that ST1.4 is able to express and assemble (9). For virion analysis, LMH cells were transfected with a helper construct, which provides core, polymerase, and second envelope proteins in trans to the mutant envelope protein-expressing plasmid. Virion assembly was analyzed according to the pronase-DNase method described by Lenhoff and Summers (17), which discriminates between enveloped virions and naked capsids. All mutants were able to produce virions to similar levels, as shown by the DNA dot blots of the peak fractions following sedimentation of enveloped particles through a 20 to 70% sucrose step gradient (Fig. 4A to D, blots below panels).

The TM1 domain and its hydrophobicity is crucial for DHBV infection. The above-described mutants were then tested for their ability to infect PDHs. We used intracellular enveloped virus particles for all of the infection studies, since this allows the use of mutants which are not exported and avoids the use of polyethylene glycol for precipitation and concentration of the virus from the media, as this agent may result in nonphysiological routes of viral entry (8). Infectivity was determined by immunofluorescence of fixed PDHs with monoclonal antibody (7C12). The L protein, seen as a doublet, represents phosphorylated and unphosphorylated forms. The asterisk indicates a glycosylated form of mutant S (9).
Panels E and F of Fig. 4 represent the mock and positive-duck-serum control infections, respectively.

The LT1.4 mutant is able to bind to the attachment receptor, CPD, and enter the hepatocyte. To determine at which stage of the entry pathway the LΔTM1 and LT1.4 mutants were affected, we assessed their abilities to bind to the attachment receptor, CPD. Binding of SVPs produced from transfected cells to recombinant CPD was assessed by cosedimentation of SVPs and any bound CPD through 20% sucrose and subsequent detection of SVP-bound CPD by Western blotting with a polyclonal antiserum to CPD (180 kDa) and a monoclonal antibody to pre-S, which detects the 36-kDa L protein. The results are shown as percentages of binding to CPD, normalized for the relative amounts of L protein detected from the pelleted SVPs. Wild-type SVPs with prior trypsin treatment, which cleaves the external pre-S receptor binding domain, had reduced CPD binding abilities, as expected (13) (Fig. 5). CPD does not sediment in the absence of SVPs, as shown in Fig. 5, control lane. The LT1.4 mutant was able to bind CPD at levels similar to that of the wild type, while no binding to CPD was detected for the LΔTM1 mutant (Fig. 5). Thus, the block to infection of LT1.4 is downstream of receptor binding, while the deletion of TM1 appears to drastically affect L folding and the pre-S–CPD interaction, rendering it noninfectious.

Although the LT1.4 mutant was shown to be able to bind to CPD, the block to infection could still result from the inability to bind to one of the proposed DHBV coreceptors (18). To provide further evidence that the infection block occurs at the stage of fusion, we monitored entry of mutant and wild-type particles in the hepatocyte by colocalization with the endosomal marker, transferrin, as done for the serum-derived virus in Fig. 2A. Image overlays of transferrin and the DHBV L protein show that the LT1.4 mutant, as well as wild-type particles,
colocalizes with transferrin after the 2-h infection period, indicating that the mutant enters the cell (Fig. 6). Immunofluorescence staining at day 7 postinfection confirmed that the wild-type but not the mutant infection progressed as seen previously for Fig. 4D (data not shown).

Alanine substitutions in a TM1 peptide cause defects in the aggregation and lipid mixing of phospholipid vesicles. To further confirm the function of L TM1 as the fusion peptide, a mutant peptide was assessed for the ability to engage in aggregation and lipid mixing of phospholipid vesicles. Unfortunately, it was not possible to synthesize a peptide that spans the entire TM1, due to the high hydrophobicity of the sequence. Hence, we used a peptide sequence spanning the region used in studies by Rodriguez-Crespo and coworkers (25–27). They have demonstrated the aggregation and lipid mixing of phospholipid vesicles with partial N-terminal TM1 peptides corresponding to TM1 sequences of all of the hepadnaviruses. The peptides encompassed the positions of the first two of the four hydrophobic residues (Ile165 and Leu176) used in this study. We used both assays to test the wild-type peptide, a scrambled peptide of the same region as a control, and the mutant peptide with alanine substitutions at Ile8 and Leu15.

The liposome aggregation assay measures the turbidity increase associated with the increase in size and clumping of the phospholipid vesicles as a result of the insertion of the fusion peptide into the lipid membranes. The results show that, irrespective of the pH conditions used, only the wild-type peptide has the ability to aggregate phospholipid vesicles, with little or no activity present for both mutant and scrambled peptides (Fig. 7A and B). In addition, comparison of the wild-type peptide activities at pH 7 and pH 5 indicates that a lower pH facilitates a more rapid reaction (Fig. 7C). A 10-min incubation window was chosen because time course experiments performed with the wild-type peptide showed that the aggregation reaction goes to completion within this time frame (data not shown).

To assess the reaction kinetics more accurately, a lipid-mixing assay was used, where the insertion of the peptide causes lipid mixing between populations of fluorescently labeled and unlabeled lipid vesicles, leading to subsequent dequenching of the R18 probe as monitored using a fluorometer. The assay was performed at both neutral and late-endosome pH conditions. Only the wild-type peptide exhibited any significant lipid-mixing activity, as shown in a representative experiment.
Moreover, as seen for the lipid vesicle aggregation experiment described above, although the peptide is able to induce lipid mixing at both neutral and acidic pHs, the kinetics of the reaction is much slower at pH 7 (Fig. 8C).

**HBV/DHBV L TM1 chimeras are able to assemble into infectious virions.** Given the conservation in general hydrophobicity of TM1 between all of the hepadnaviruses, we speculated that this region plays the role of a fusion peptide for all family members. To ascertain the possibility that the sequence corresponding to HBV TM1 can be functionally interchanged with that of DHBV, an L DHBV chimera carrying the HBV TM1 sequence was created (L: DHTM1) (Fig. 9A). The chimera was tested for expression and assembly into SVPs and virions. The L: DHTM1 chimera was able to assemble into SVPs although with little apparent incorporation of the chimeric L chain (Fig. 9B, lane 3). Particles containing chimeric L were unable to be exported to the media (Fig. 9B, lane 5).

Previous studies using influenza hemagglutinin chimeras with the HBV TM1 fragment in place of its fusion peptide have shown that Gln<sup>177</sup> appears critical for the folding of the region and that influenza fusion was restored only after this residue was mutated to a hydrophobic residue, isoleucine (1). An additional chimera (L: DHTM1 Q177L) carrying a similar substitution using the corresponding DHBV residue, Leu<sup>177</sup>, in the TM1 sequence similarly improved expression and assembly of SVPs and restored export (Fig. 9B, lanes 2, 4, and 6). Both chimeras produced enveloped virions (Fig. 9C). The analysis of chimeras for infectivity on PDHs showed both were infectious, indicating that either TM1 sequence is acceptable for DHBV fusion (Fig. 9C).

**DISCUSSION**

While it has been established that DHBV enters the cell via receptor-mediated endocytosis (2, 3, 14), it has not been es-
established whether DHBV requires passage through the acidic endosome pathway. In this study, we have examined DHBV endocytosis and whether TM1, the domain exposed following low-pH treatment of particles, plays an essential role in the entry process. Bafilomycin A1 blocked early- to late-endosome traffic as well as raising endosomal pH and was effective in completely inhibiting DHBV entry during the 2-h infection period (Fig. 2B). Previously published studies with lysosomotropic agents, which raise endosomal pH, showed a block to infection of Semliki Forest virus but not of DHBV (14, 24). Since Semliki Forest virus, which has a pH threshold of 6.5, was inhibited in these studies, it can be assumed that the pH of the early endosome (pH 6.2) was effectively raised to neutrality. We therefore conclude that DHBV fuses in the late and not the early endosome.

The kinetics of DHBV entry is slow, with internalization taking 2 to 3 h followed by a protracted period of approximately 14 h before nuclear import of the viral genome is detected (6, 14). Although we used a 1-h incubation at 4°C followed by a 2-h incubation at 37°C in our transferrin colocalization study, very few particles were actually observed inside the cell. However, this is consistent with the attachment receptor being a Golgi-resident protein with little expression on the cell surface, and previous findings that have shown that only a very small percentage (0.05%) of input particles are able to bind to PDHs (6). It can also be assumed that the observed virus has been internalized, as the findings of Funk and co-workers have also demonstrated that most of the bound particles are internalized within 2 h of incubation (6).

Similarly, for the bafilomycin A1 study, the period of 2 h when cells were exposed to virus is sufficient time for internalization of most of the attached particles from the cell surface. Thus, the very few positive cells detected at the 2-h time point addition of the drug and the small increase after the 4-h time point addition suggest that once endocytosed, virions have a relatively long transit through the endocytic pathway (Fig. 2B). The block seen in early- to late-endosomal trafficking facilitated by bafilomycin A1 is also consistent with the finding that DHBV has a microtubule-sensitive stage in the 4-h postinternalization, which is reversible upon removal of the drug nocodazole up to that time point and no longer (6). Accordingly, virions are still competent for exit from the endosome compart-
ment after exposure to low pH and other endosomal conditions for 4 h, suggesting that the kinetics for the conformational change in the envelope and exposure of the fusion peptide may be very slow.

The exposure of the hydrophobic TM1 domain enables binding to lipid membranes, seen as one of the first steps in virus fusion. We have established that the hydrophobicity of TM1 of L and not that of S is crucial for DHBV infectivity by substitution in TM1 of four leucine and isoleucine residues with alanines (Fig. 4). This LT1.4 mutant was able to bind to the receptor, CPD (Fig. 5), and colocalize with the endosomal marker, transferrin (Fig. 6), indicating that the block to infection occurred downstream of these early entry events. Taken together, these data suggest that the block to infection of the LT1.4 mutant occurs at the fusion stage, with the TM1 domain acting as a fusion peptide.

The importance of the hydrophobicity of L TM1 and its role as the DHBV fusion peptide are further supported by liposome aggregation and mixing studies of synthetic alanine mutant and wild-type peptides. The mutant peptide with lowered hydrophobicity was shown to be defective in both liposome aggregation and lipid mixing. The correlation between defects in the lipid perturbation properties of a synthetic fusion peptide sequence and fusion protein dysfunction has been shown previously with influenza haemagglutinin (22). Hence, it can be assumed that the block in infection of the LT1.4 mutant is caused by the display of a defective fusion peptide sequence. The properties of the peptides used provide a further insight into the nature of the TM1 fusion peptide. Liposome aggregation and lipid mixing were demonstrated with the wild-type sequence peptide but not with the scrambled sequence peptide. The scrambled peptide has the same hydrophobicity and a hydrophobic-residue distribution similar to that of the wild-type peptide but differs in the distribution of polar residues. Accordingly, the secondary-structure prediction algorithm (28) indicates that the scrambled peptide may be folded differently from the wild-type and mutant peptides. This implies that the lipid perturbations induced by the TM1 peptide are dependent not only on the hydrophobicity but also on the sequence of residues defining the folding of this region.

Furthermore, the kinetics of liposome aggregation and mixing of the wild-type sequence peptide were shown to be pH dependent. Although the wild-type peptide was able to induce both processes regardless of pH conditions tested, the reactions were much more rapid at low pH. In contrast to the results obtained by Rodríguez-Crespo and coworkers (26), our results indicate that low pH, although not crucial, allows for a more rapid reaction. This discrepancy can be explained by the 1-h endpoint used in those studies, whereas our results have shown that the reaction is complete within 10 min and that the kinetic differences are only visible within this time window.

The apparent nonessential role of pH is consistent with previous studies using lysosomotrophic agents (14, 24) and is also supported by the fact that a conformational change that exposes the fusion peptide is also not dependent strictly on the low pH encountered in the late endosome, since such a change also occurs at pH 6.5 under reducing conditions. Since weak bases may not raise the pH of the late endosome to neutrality, these mild pH conditions may have been present and could account for the lack of inhibition seen in those previous studies. The combined effect of a reducing agent and low pH on L conformation is reversible, and virions remain infectious upon pretreatment, in contrast to low pH treatment alone, where virions become inactivated (10). This intermediate, reversible conformation would suggest that another factor(s) may be necessary to trigger the final fusion conformation. A likely candidate factor would be a protease for liberation of the fusion peptide for N-terminal exposure and insertion into the endosomal membrane. Studies by Lu and coworkers (19, 20) showed that V8 protease cleavage upstream of TM1 followed by low pH treatment enabled HBV infection of HepG2 cells. It is the only evidence so far that suggests a role for a protease in the virus entry, although this particular example may not be representative of the natural entry mechanism. The potential requirement for protease cleavage within the virus fusion compartment is an unusual strategy in enveloped viruses and may contribute to the virus host specificity requirements of the hepadnaviruses. Indeed, a recent study has shown that the Ebola virus envelope glycoprotein requires endosomal cyto- steine proteases to trigger fusion (5).

This study provides a new insight into the entry of DHBV, identifying TM1 and its hydrophobicity as essential for fusion. Following receptor attachment and endocytosis, the virus is targeted to the late endosome, where the L protein undergoes a conformational change facilitated by low pH and deforming conditions of this compartment. The conformational change exposes the fusion peptide (TM1) and allows it to anchor and destabilize the endosome lipid bilayer. In the current topological model, this conformational change would require the removal of TM1 from a lipid bilayer (Fig. 1E). However, it is possible that this domain is initially present on the virus surface but sequestered by the pre-S domain until the conformational change occurs, exposing the fusion peptide in a manner similar to that of other enveloped viruses. Accordingly, if this model is correct for the external L topologies and given that TM1 of the internal L topology is not inserted in the endoplasmic reticulum membrane (32) (Fig. 1C), then one can conclude that this hydrophobic domain is not primarily a transmembrane domain.

The ability of virions with chimeric L chains carrying HBV TM1 to infect PDHs shows that domain to be functionally interchangeable between the viruses in terms of infection. Although the folding in this region may differ between DHBV and HBV, as illustrated by the need to mutate a nonconserved Gln (27) residue in order to restore particle export, it does not affect the ability of the chimera to infect PDHs. This suggests that the TM1 domain of HBV may also act as a fusion peptide, thus implicating that the same region is involved in the fusion of all of the hepadnaviruses. However, this would have to be confirmed by an analogous chimera in HBV and infection of primary human hepatocytes.

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REFERENCES

tinin with a putative fusion peptide from hepatitis B virus. Virus Res. 68: 35–49.